Immunological identification of four different polypeptides in 'subunit VII' of mammalian cytochrome *c* oxidase

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Rat liver cytochrome *c* oxidase was separated by SDS-gel electrophoresis into 13 polypeptide bands. Monospecific antisera against the isolated polypeptides VIIa, VIIb and VIIc were raised in rabbits. Cytochrome *c* oxidase was blotted on nitrocellulose and incubated with the antisera. The antisera reacted only with their corresponding polypeptides, indicating no immunological relationship between polypeptides VIIa, VIIb and VIIc. The data also exclude that these polypeptides are proteolytic breakdown products of larger subunits.

Cytochrome c oxidase *Immunoblotting* *Subunit composition* *Monospecific antiserum* *SDS-gel electrophoresis*

1. INTRODUCTION

Cytochrome *c* oxidase, the terminal enzyme of the respiratory chain of mitochondria and some aerobic bacteria, is composed of several subunits, the exact number depending on the evolutionary stage of the organism. Two to three subunits have been identified in bacteria, 7–8 in fungi and 12–13 in mammalian tissues (review [1–4]). In eucaryotic cells 3 polypeptides are of mitochondrial origin; the remainder are determined by the nuclear genome. The number of functional subunits which comprise the mammalian cytochrome *c* oxidase complex is still a subject of debate [5–7] due to the difficulty of separating all polypeptides and due to the lack of a known role for the 9–10 nuclear coded polypeptides [2,7]. With a standard SDS-gel electrophoretic separation system mammalian cytochrome *c* oxidase is only separated into 7 bands [1]. We have described an SDS-gel electrophoretic system [8] which separates subunit V, 'subunit VI' and 'subunit VII' into 2, 3 and 4 different polypeptide bands, respectively, yielding a total of 13 distinct bands. For 12 polypeptides of the beef heart enzyme, isolated by column chromatography, the complete amino acid sequence has been described [9]. The identity of these polypeptides with 12 distinct gel bands was obtained by N-terminal amino acid sequence analysis of the extracted gel bands [2,7].

Although different N-terminal amino acid sequences have been identified for the 4 small polypeptides of pig liver and heart cytochrome *c* oxidase subunit VII (Phe-Glu-Asn, Ser-Gly-Tyr, Ser-His-Ser and Ile-His-Ser for pig liver polypeptides VIIa, VIIb, VIIc and VIII, respectively) [7], it could not be excluded that one or more of the polypeptides might represent a proteolytic breakdown product of a larger subunit. This possibility was eliminated for polypeptide VIII by application of a monospecific antiserum [11]. Polypeptides VIIa and VIIc could be identified with Buse and coworkers' subunits VIIIc and VIIIa (their nomenclature), for which the complete amino acid sequences have been obtained [9]. Only polypeptide VIIb has not been found by other investigators.

Here, 3 different and monospecific antisera against polypeptides VIIa, VIIb and VIIc have been raised and used to demonstrate the immunological difference of these polypeptides. No cross reactivity with other polypeptide bands was

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detected on the immunoblot of electrophoretically separated rat liver cytochrome *c* oxidase.

2. MATERIALS AND METHODS

Nitrocellulose BA 85 (pore size 0.45 μM, 20 × 16 cm) was obtained from Schleicher and Schüll (Dassel) and FITC-protein A from Pharmacia (Freiburg).

Rat liver cytochrome *c* oxidase was isolated from mitochondria with nonionic detergents as in [12] but the gel filtration step was omitted. The ammonium sulfate-precipitated enzyme was dialyzed against distilled water and stored at −70°C. The isolated enzyme contained 7.6 nmol heme *a*/mg protein. The absorbance ratio 280/420 nm of the isolated enzyme ranged from 2.4 to 2.8.

Polypeptides VIIa, VIIb and VIIc were isolated from cytochrome *c* oxidase after separation by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) [8]. Maximally 1.2 mg protein was applied to each slab gel (16 × 23 × 0.15 cm). The stained bands were cut out and the protein was extracted either with SDS-buffer as in [13], or with 30 vols of 66% acetic acid [14]. The concentrated acetic acid extract (2 ml) was purified by gel chromatography on a Bio-Gel P-4 column (100–200 mesh, 1.8 × 21 cm) in 66% acetic acid. The protein fraction was concentrated and stored at −20°C. The yield of extracted protein ranged from 20 to 30%. Antisera were raised in rabbits as in [13] by two intramuscular injections of 200 μg polypeptides, isolated with SDS-buffer. Booster injections (50–100 μg subcutaneous) were repeated twice.

The electrophoretic transfer of proteins from slab gels to nitrocellulose [15–17] was done as in [11]. The electrophoretic buffer solution was 150 mM glycine, 20 mM Tris, 20% methanol (pH 8.3). The current was 220 mA for 20 h. After protein transfer, the nitrocellulose was stained with 0.025% amido black in 22.5% ethanol, 7.5% acetic acid. One lane was separately fixed and destained with 7.5% acetic acid, 5% ethanol. From the other lanes the stain was completely removed by incubation with 5% bovine serum albumin in 10 mM Tris–HCl (pH 7.5), 0.9% NaCl (TS buffer) overnight. Subsequent incubation with the antisera, diluted 1:5 in TS, was for 24 h, followed by washing 3 times with 1% Triton X-100 in TS and once with TS. The incubation with FITC-protein A (0.1 mg/200 ml TS) was performed for 2 h, followed by two washings with 1% Triton X-100 in TS and one with TS. Immunofluorescence was photographed as in [11].

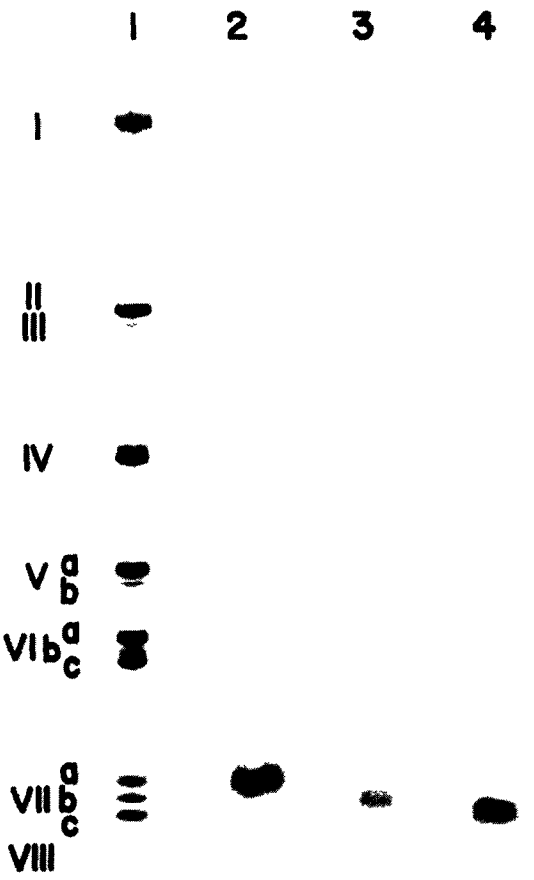

Fig.1. SDS-gel electrophoresis of rat liver cytochrome *c* oxidase and of isolated polypeptides VIIa, VIIb and VIIc. The lower band of polypeptide IV represents a proteolytic breakdown product of the upper band [13]. The polypeptides were extracted from gel bands with 66% acetic acid as described in section 2. Lane 1, 33 μg isolated cytochrome *c* oxidase; lane 2, 1.4 μg polypeptide VIIa; lane 3, 3.2 μg polypeptide VIIb; lane 4, 0.7 μg polypeptide VIIc. The weak staining of polypeptide VIIb is due to aggregation and partial loss of the isolated protein in 66% acetic acid. The indicated amount of protein was determined immediately after gel chromatography (see section 2).

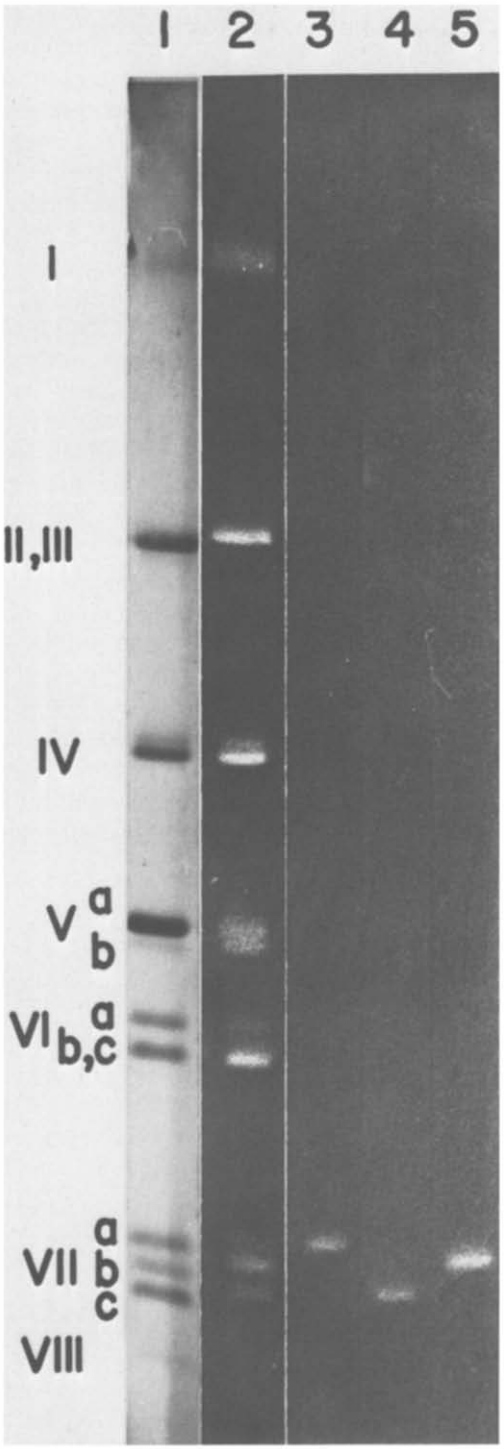

Fig.2. Monospecific antisera against polypeptides VIIa, VIIb and VIIc do not crossreact with other polypeptides of isolated cytochrome *c* oxidase. Cytochrome *c* oxidase was separated by SDS-gel electrophoresis, transferred to nitrocellulose, stained with amido black (lane 1), destained and immunodetected with antisera against holocytochrome *c* oxidase (lane 2), polypeptide VIIa (lane 3), polypeptide VIIc (lane 4) and polypeptide VIIb (lane 5) as described in section 2.

3. RESULTS

Mammalian cytochrome *c* oxidase, as isolated under standard conditions, can be separated by SDS-gel electrophoresis into 13 polypeptide bands. This was shown for the enzyme from rabbit liver, beechmarten liver [12], rat liver, heart and kidney [11,18], bovine liver and heart [19], pig heart [8], liver, kidney and diaphragm (in preparation) and human heart (in preparation). In fig.1 the SDS-gel electrophoretic polypeptide pattern of isolated rat liver cytochrome *c* oxidase is shown together with the purified polypeptides VIIa, VIIb and VIIc which were extracted from the stained bands of preparative gels with 66% acetic acid. Antisera against the purified polypeptides extracted with SDS-buffer were raised in rabbits.

The specificity of the antisera is demonstrated by an immunoblot ('western blot') as shown in fig.2. After the electrophoretic transfer of separated cytochrome *c* oxidase onto nitrocellulose, several lanes of the sheet were separately incubated with antisera against holocytochrome *c* oxidase, polypeptide VIIa, VIIb or VIIc, and the immunoreactive bands were visualized with FITC–protein A. Whereas the antiserum against the holoenzyme reacts with all polypeptides except VIc and VIII (but in some cases a weak reaction was found), the monospecific antisera react exclusively with the corresponding polypeptides. This suggests that polypeptides VIIa, VIIb and VIIc represent 3 non-related proteins which are not proteolytic breakdown products of larger subunits.

4. DISCUSSION

In contrast to soluble enzyme complexes, the subunit composition of hydrophobic membrane complexes is difficult to determine if subunits of similar molecular mass are present. Isoelectric focussing, successfully applied to soluble protein

complexes, does not give satisfactory results with hydrophobic proteins. The best separation to date has been obtained by SDS-gel electrophoresis in the presence of urea. The small polypeptides of cytochrome *c* oxidase (M_r 5000–12000) can only be separated if the acrylamide concentration is high (18–19%) and the pH of the electrode buffer and separation gel is specifically adjusted for different species and tissue types of cytochrome *c* oxidase.

Our aim was to substantiate the existence of 4 different polypeptides of M_r 5000–6200 [7] which are not resolved by SDS-gel electrophoresis under standard conditions. The results clearly demonstrate that polypeptides VIIa, VIIb and VIIc are 3 immunologically different proteins which are unrelated to any other polypeptide of cytochrome *c* oxidase. The same conclusion was obtained for polypeptide VIII in [11].

The N-terminal amino acid sequence of polypeptide VIIb is different from that of all other nuclear-coded polypeptides [7]. The isolated protein easily tends to aggregate and therefore has not been found by other groups [9]. Since this polypeptide has been shown to occur in stoichiometric amounts [19], it can be considered an intrinsic polypeptide of mammalian cytochrome *c* oxidase which occurs in addition to the 12 polypeptides with known amino acid sequence [9].

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